

Cellular Immune Responses in Humans Induced by Two Serogroup B Meningococcal Outer Membrane Vesicle Vaccines Given Separately and in Combination

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MenBvac and MeNZB are safe and efficacious outer membrane vesicle (OMV) vaccines against serogroup B meningococcal disease. Antibody responses have previously been investigated in a clinical trial with these two OMV vaccines given separately (25 µg/dose) or in combination (12.5 and 12.5 µg/dose) in three doses administered at 6-week intervals. Here, we report the results from analyzing cellular immune responses against MenBvac and MeNZB OMVs in terms of antigen-specific CD4⁺ T cell proliferation and secretion of cytokines. The proliferative CD4⁺ T cell responses to the combined vaccine were of the same magnitude as the homologous responses observed for each individual vaccine. The results also showed cross-reactivity in the sense that both vaccine groups receiving separate vaccines responded to both homologous and heterologous OMV antigen when assayed for antigen-specific cellular proliferation. In addition, a multiplex bead array assay was used to analyze the presence of Th1 and Th2 cytokines in cell culture supernatants. The results showed that gamma interferon, interleukin-4 (IL-4), and IL-10 responses could be detected as a result of vaccination with both the MenBvac and the MeNZB vaccines given separately, as well as when given in combination. With respect to cross-reactivity, the cytokine results paralleled the observations made for proliferation. In conclusion, the results demonstrate that cross-reactive cellular immune responses involving both Th1 and Th2 cytokines can be induced to the same extent by different tailor-made OMV vaccines given either separately or in combination with half the dose of each vaccine.

Neisseria meningitidis serogroup B vaccines based on outer membrane vesicles (OMVs) from defined serogroup B strains have been shown to be efficacious to control clonal outbreaks in several countries, including Norway, Cuba, and New Zealand (1–3). The PorA protein is the immunodominant antigen in OMV vaccines and elicits protective immune responses (4). PorA shows large sequence variation between strains, and a limitation of OMV vaccines is that they induce mainly strain-specific antibodies (5), but cross-protective antibodies against other B strains have been observed in small children and adults (3, 5). The OMV vaccine MenBvac was developed based on the B:15:P1.7,16 strain representative of the previous meningococcal epidemic in Norway. Based on several clinical trials, this vaccine has been shown to be safe, immunogenic, and to confer protection against meningococcal B disease (6–9). MenBvac has also recently been used to combat an outbreak of serogroup B disease in France demonstrating that OMV vaccines can be efficient against heterologous B strains provided that they are sufficiently immunologically close (10, 11).

A similar meningococcal serogroup B OMV vaccine (MeNZB) based on a different strain (B:4:P1.7-2,4) was developed and introduced in 2004 to control the meningococcal epidemic in New Zealand (12–14) showing more than 70% effectiveness (3). However, in most geographical regions with endemic serogroup B outbreaks, several different clones are responsible for disease, and the ideal vaccine should therefore elicit protection against a broad range of clinical strains in all age groups. This may principally be achieved by combining OMVs from different serogroup B strains or include cross-protective antigens (1, 6, 15–17). Importantly, preclinical studies in mice have suggested that half the normal antigen dose of each OMV vaccine could elicit similar immune responses compared to full doses when administered in combination (16) and that sequential immunization with heterologous

OMV strains could elicit broadly protective serum antibodies (17). The safety profile and immunogenicity of a combined OMV vaccine, consisting of MenBvac and MeNZB (half dose each) adsorbed to aluminum hydroxide, were then tested in a clinical trial design consisting of three primary doses given with 6-week intervals and a fourth booster-dose given 1 year later (18). With respect to antibody responses measured as serum bactericidal activity (SBA), opsonophagocytosis, and enzyme-linked immunosorbent assay (ELISA), the results showed that the immune responses to the combined vaccines were of the same magnitude as the homologous responses observed in control groups receiving individual vaccines (18). In addition, the safety profile of the combined vaccine was not different from those previously seen after the administration of separate monovalent vaccines (1, 6, 9). Although this work contributed to the concept of combining OMV vaccines to cover a broader range of epidemic strains, important steps toward improved epidemic coverage in different age groups has thereafter been taken by the development of a multicomponent serogroup B vaccine consisting of MeNZB OMV admixed with recombinant antigens (4CMenB) (19–21). Although such vaccines have been

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shown to induce protective antibody responses with broadened strain specificity, information on cellular immune responses supporting antibody-mediated effector functions is lacking.

Whereas protection against extracellular bacterial infections is mainly mediated by functional antibodies, measured as SBA and opsonophagocytosis, CD4⁺ T cells also play an important role in Th2 cytokine-mediated help to B cells with regard to immunoglobulin class switch, affinity maturation, and increasing the magnitude of the antibody response (22, 23). T cells are also necessary for the induction of immunological memory and can indirectly contribute to the uptake and destruction of bacteria by activating phagocytes. To evaluate T cell responses to single and combined OMV vaccines, we tested peripheral blood mononuclear cells (PBMCs), sampled from the same clinical trial as described above (18), for antigen-specific T cell proliferation and cytokine secretion. Consistent with the results for functional antibody responses, we report here that all vaccine groups showed a T cell response against both vaccine antigens used and that the combined vaccine preparation induced a cellular immune response comparable to that seen after administration of individual vaccines. Hence, these results on cellular immunity support the concept of combining different OMV vaccines for covering meningococcal B epidemics caused by different bacterial strains.

MATERIALS AND METHODS

Vaccines. MenBvac was manufactured at the Norwegian Institute of Public Health (NIPH) from a B:15:P1.7,16 meningococcal strain (44/76) by fermentor growth and extraction of the OMVs with the detergent deoxycholate. OMVs were purified by fractionated centrifugation and adsorbed to aluminum hydroxide (24). MeNZB was prepared at the NIPH by a similar technique from a B:4:P1.7-2,4 meningococcal strain (NZ98/254) (18). This strain was kindly provided by Diana Martin at the Institute of Environmental Science and Research in New Zealand. One dose (0.5 ml) of either MenBvac or MeNZB contained 25 µg of outer membrane protein and 1.67 mg of aluminum hydroxide (corresponding to 0.57 mg of aluminum). The combined vaccine (0.5 ml) contained 12.5 µg of OMVs from MenBvac and 12.5 µg of OMVs from MeNZB, and the same amount of aluminum hydroxide as either of the two separate monovalent vaccines. The combined vaccine was prepared by mixing MenBvac and MeNZB immediately before injection.

Study participants. Healthy adults who had given written informed consent prior to study entry and who fulfilled all inclusion criteria and none of the exclusion criteria (e.g., pregnancy, chronic disease, previously having received meningococcal B vaccine of any kind or previous disease caused by *N. meningitidis*) were eligible for participation in the study. The subjects were mainly recruited among students in the Oslo area. This study was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonization guideline for Good Clinical Practice and other national legal and regulatory requirements. The trial was approved by the Regional Committee for Medical Research Ethics and the Norwegian Medicines Agency.

Administration and immunization schedule. Three primary vaccine doses were administered at weeks 0, 6, and 12, and a booster dose was administered 1 year later. In the primary schedule (part A), the subjects were randomly assigned into three groups (30 participants in each group) to receive either the combined MenBvac-MeNZB vaccine, MenBvac only, or MeNZB only. For the booster dose (part B), subjects receiving MenBvac or MeNZB alone in the primary three-dose schedule were randomized (1:2 ratio) to receive either the same monovalent vaccine as used in the primary schedule or the other monovalent vaccine (crossover schedule), while all subjects receiving the combined vaccine within the primary schedule also received the combined vaccine as a booster (18). All vaccines

were administered intramuscularly in the deltoid region of the nondominant arm.

We present here an analysis of the available PBMC samples from part A with respect to antigen-specific T cell proliferation and cytokine secretion. The number of vaccinees included in the analysis ($n = 12$ to 22) is lower than the number of recruited participants. This was due to withdrawal from the study, limited blood volume, low yield of PBMCs after isolation, or failure of positive- or negative-control stimulation in the assays. Only pairwise and valid results for the same participant before and after vaccination were included.

Blood samples and PBMC isolation. Venous blood collected in ACD vacutainers (Becton Dickinson, United Kingdom) was obtained before vaccination and 6 weeks after the third vaccine dose (part A). PBMCs were isolated from whole blood by density centrifugation (Lymphoprep; Amersham, United Kingdom) and cryopreserved in liquid nitrogen according to standard procedures (10% dimethyl sulfoxide and 25% fetal calf serum).

T cell proliferation assay. PBMC samples from both visits were thawed and assayed by the [³H]thymidine incorporation method for proliferative responses to optimal concentrations of the OMVs from the MenBvac and MeNZB vaccines, as well as to *Mycobacterium bovis* BCG (irrelevant antigen) and PHA (phytohemagglutinin; mitogen) for control purposes. OMV antigens (4 µg/ml), BCG (20 µg/ml), or mitogen (5 µg/ml) in triplicate and PBMCs (100,000 cells/well) suspended in RPMI 1640 (with 15% human AB serum, glutamine, penicillin, and streptomycin) was plated in flat-bottom 96-well microtiter plates (Costar, USA) with a final volume of 200 µl/well. After 6 days of incubation in 5% CO₂ at 37°C, the cells were pulsed with [³H]thymidine (1.3 µCi/well; Amersham, United Kingdom) for 4 h, harvested (Packard FilterMate), and incorporated thymidine was determined by liquid scintillation counting (Packard TopCount). The CD4⁺ phenotype of proliferating cells on day 6 has previously been confirmed by flow cytometry (25). In addition, it was possible to obtain a dose-dependent inhibition of proliferation by adding blocking anti HLA-DR antibodies (B8.11) to the assay (25). Proliferative T-cell responses are expressed as delta counts-per-minute (cpm) values calculated by subtracting the mean of triplicate cpm values obtained in the absence of antigen (cpm medium) from the mean of triplicate cpm values obtained in the presence of antigen (cpm Ag).

Multiplex cytokine bead array assay. A bead array kit for flow cytometric measurements of human Th1 and Th2 cytokines (gamma interferon [IFN-γ], interleukin-2 [IL-2], tumor necrosis factor alpha [TNF-α], IL-4, IL-5, and IL-10) was used to analyze OMV-induced cytokine secretion into cell culture supernatants collected before cell harvesting (day 6) of the proliferation assays (CBA human Th1/Th2 cytokine kit; Becton Dickinson, United Kingdom). The bead array assay was run according to instructions from the manufacturer, and the amount of cytokines was calculated based on recombinant standards for individual cytokines. IFN-γ results were expressed as arbitrary units (AU).

Statistical methods. *P* values for the Wilcoxon paired signed-rank test (comparison of pre- and postresponses) and the Mann-Whitney test (comparison between different vaccine groups) were determined by the use of Prism software (GraphPad Software, La Jolla, CA).

RESULTS

The complete design of the vaccine trial, including three primary immunizations (part A) and one booster dose (part B), the inclusion of participants, the safety profile, and the antibody responses, has previously been published (18). We describe here the exploratory analysis of PBMC samples from part A with respect to antigen-specific T cell proliferation and cytokine secretion.

Proliferative T cell responses. Frozen PBMC samples were thawed, counted, and assayed for antigen-specific CD4⁺ T cell proliferation by measuring thymidine incorporation at day 6. In addition to stimulation with OMVs, representing the two vaccines used, cells were also stimulated with BCG and PHA for positive-

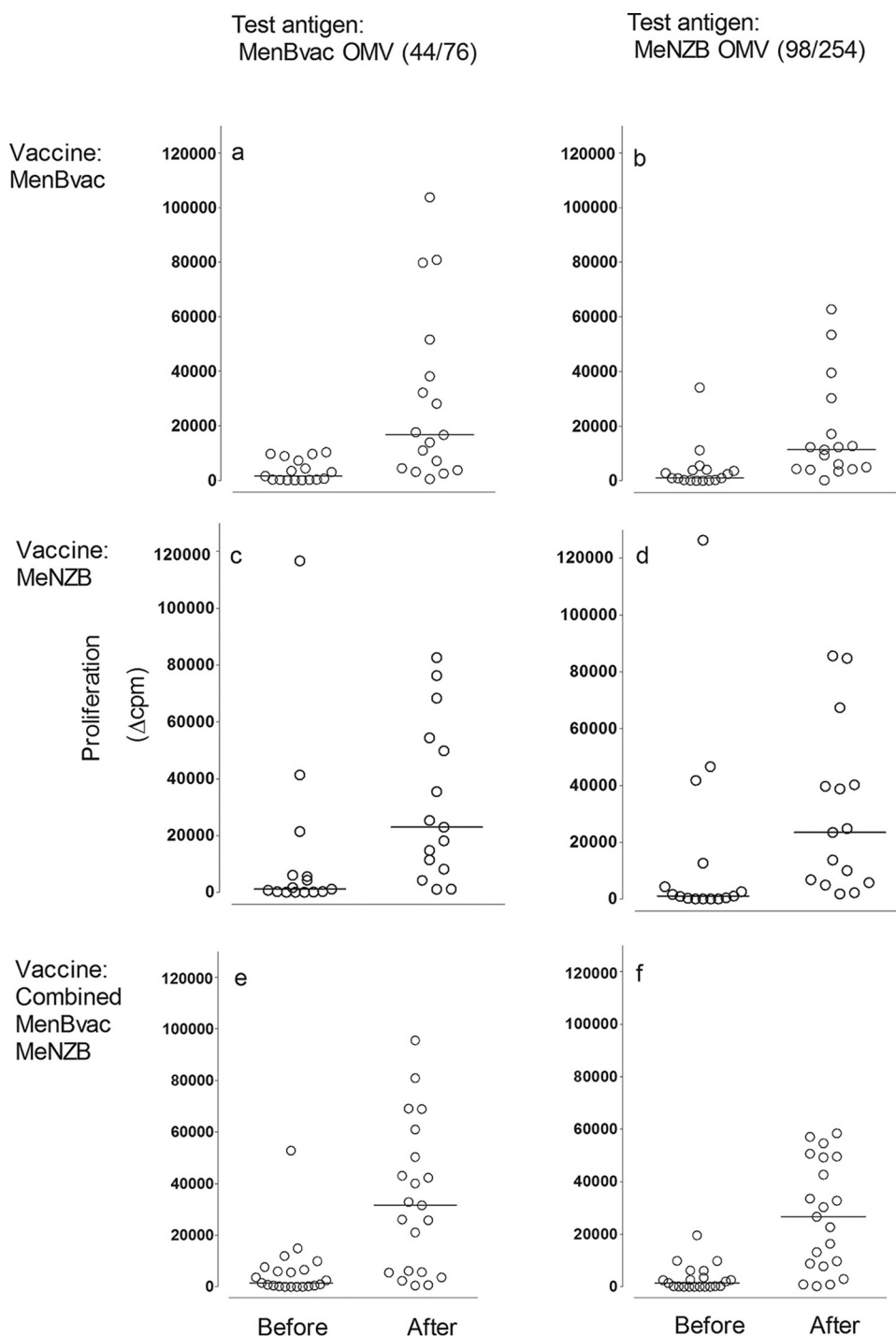


FIG 1 Proliferative T cell responses (delta cpm) *in vitro* against MenBvac and MeNZB OMVs before (week 0) and 6 weeks after the third dose of vaccination with MenBvac alone ($n = 17$), MeNZB alone ($n = 15$), or combined vaccine ($n = 21$). The P values according to the Wilcoxon paired signed-rank test for vaccine-induced proliferative responses observed in the different vaccine groups were 0.0011 (a), 0.0029 (b), 0.0103 (c), 0.0554 (d), 0.0002 (e), and 0.0002 (f).

control purposes. [Figure 1](#) shows the results from testing all three vaccine groups for proliferative responses with both MenBvac (44/76) OMVs and MeNZB (98/254) OMVs before immunization (first dose) and 6 weeks after the third dose. The median preimmunization value in all groups demonstrated a nonresponder status without any differences in response to the OMV antigens

(range, 1,000 to 1,580 cpm). MenBvac vaccinated participants showed a significant response to both the vaccine antigen given and MeNZB OMVs ([Fig. 1a](#) and [b](#), median values of 16,700 and 11,390 cpm, respectively). Participants immunized with MeNZB also responded to both vaccine antigens ([Fig. 1c](#) and [d](#), median values of 23,480 cpm in both cases). Consistent with this, partici-

pants receiving the combined vaccine showed a significant increase in antigen specific T cell proliferation to both OMV preparations (Fig. 1e and f, median values of 31,540 cpm for 44/76 and 26,600 cpm for 98/254). Although the recipients of the combined vaccine showed the highest median response values, there was no significant difference between the vaccine groups, and this was the case for both OMV antigens tested. Similarly, within all three groups, the median postimmunization responses were in the same range for both test antigens used. No significant changes in proliferative responses to the irrelevant vaccine antigen BCG or the mitogen PHA was observed (results not shown).

Cytokine responses. A bead array kit for flow cytometric measurements of human Th1 and Th2 cytokines was used to analyze OMV-induced cytokine secretion into cell culture supernatants harvested from the proliferation assays described above (same stimulation with antigens and controls). Figure 2, 3, and 4 show the results from analyzing all three vaccine groups for antigen-induced secretion of IL-10, IL-4 (Th2), and IFN- γ (Th1), respectively, after *in vitro* stimulation with MenBvac and MeNZB OMVs. For all cytokines, the preimmunization levels in all vaccine groups were similar independently of the antigen used for stimulation of cells (52 to 75 pg/ml for IL-10, 20 to 25 pg/ml for IL-4, and 0.7 to 1.5 AU/ml for IFN- γ).

For both the MenBvac- and MeNZB-vaccinated groups the results showed significant increases in IL-10 secretion when cells were stimulated with both vaccine antigens *in vitro* (Fig. 2a, b, c, and d). The postimmunization levels for IL-10 were similar for these vaccine groups (121 to 137 pg/ml), regardless of the OMV antigen used in the assays. Consistent with this, participants receiving the combined vaccine also showed a significant increase in IL-10 secretion in response to both OMV preparations. In this group, the median levels of IL-10 detected after immunization (73 and 75 pg/ml) were lower than for the participants receiving separate vaccines (121 to 137 pg/ml), but this difference was not significant. Consistent with the results for IL-10, we also observed an increase in median values for IL-4 in all vaccine groups (Fig. 3). However, the vaccine-induced IL-4 responses were, in general, weaker than those observed for IL-10 and did not reach statistically significant levels for MenBvac (Fig. 3a and b). Measurements of IL-2, IL-5, and TNF- α in the same supernatants did not show any vaccine-induced increase in median values (results not shown). However, with respect to IL-2, IL-4, IL-5, and TNF, their response levels may be underscored, due to the fact that the optimal kinetics for detection of these cytokines is reported to be earlier than day 6 (26, 27).

Both MenBvac- and MeNZB-vaccinated participants showed an increase in median values for IFN- γ secretion when the cells were stimulated with either homologous or heterologous OMV antigen (Fig. 4a, b, c, and d). Although heterologous OMV stimulation in these groups showed a 5-fold increase after vaccination, the differences were not statistically significant (Fig. 4b and c). The postimmunization levels for the IFN- γ responses were in all cases in the range of 3.8 to 6.0 AU/ml. Participants receiving the combined vaccine showed a significant increase in the IFN- γ response after stimulation with both OMV antigens (Fig. 4e and f). However, the median IFN- γ responses levels in this group (2.7 to 3.0 AU/ml) were lower than for the groups receiving separate OMV vaccines (3.8 to 6.0 AU/ml). This situation was similar to the results we obtained for IL-10 secretion, but the difference was not significant.

DISCUSSION

The development of improved meningococcal B vaccines with increased long-term immunity in all age groups against a wide range of epidemic strains has been an important goal (3, 19, 28). In this context, OMV vaccines have played a central role in the clinical developmental process either by combining OMVs from different epidemic strains (18, 28–31) or by adding selected key antigens to OMVs derived from a dominant strain (20, 21). According to such principles, a multicomponent serogroup B vaccine (4CMenB), containing three recombinant proteins (fHbp, NadA, and NHBA) added to MeNZB OMVs (98/254), has been developed and recently licensed (20, 21). Although such vaccines have been proven to induce protective levels of functional antibody responses (i.e., the SBA) with broad strain specificity in different age groups (18–21), information on accompanying T cell responses is lacking.

We describe here cellular immune responses induced by immunization with two well-characterized meningococcal serogroup B OMV vaccines (MenBvac and MeNZB), administered separately and in combination. Design, safety profile, and antibody responses have previously been reported for this clinical trial (18). The results from ELISA, SBA, and opsonophagocytic activity (OPA) studies showed that the combined vaccine was immunogenic with regard to both vaccine strains and that functional antibody responses were of the same magnitude as the homologous responses observed for individual vaccines, even though the vaccine dose was reduced by half. Consistent with the antibody responses, we demonstrate here that cellular immune responses, measured as OMV-specific T cell proliferation and Th1 and Th2 cytokine secretion, was induced as a result of vaccination with both the MenBvac and the MeNZB vaccines given separately, as well as in combination. After immunization with the combined vaccine, both proliferative CD4⁺ T cell responses and cytokine production were of the same magnitude as the homologous responses observed for the individual vaccines. The results also showed cross-reactivity in the sense that both vaccine groups receiving separate vaccines responded in the same range to both homologous and heterologous OMV antigen when analyzed in T cell assays.

Whereas protection against extracellular bacteria is mainly mediated by functional antibodies, measured as SBA, T cells also play an important role by providing Th2 cytokine-mediated regulation of B cell responses with respect to immunoglobulin class switch and affinity maturation, as well as increasing the magnitude of the antibody response (22, 23). The importance of this is highlighted by the successful development and introduction of conjugate vaccines against pneumococcal and meningococcal disease providing effective T cell help (32, 33). Activation of T cells is necessary for the establishment of immunological memory (34), and they also facilitate the uptake and destruction of meningococci by phagocytic cells partly promoted by Th1 cytokine-mediated mechanisms (25). Extracellular bacteria, such as meningococci, will mainly be presented to CD4⁺ T cells in the context of major histocompatibility complex class II molecules, after uptake and processing by professional antigen-presenting cells. Consistent with this, we used an *in vitro* assay that detects antigen-specific proliferation of CD4⁺ T cells, as proven by flow cytometric determination of this surface phenotype and blocking of the response by using anti-HLA-DR antibodies (25, 35). We have pre-

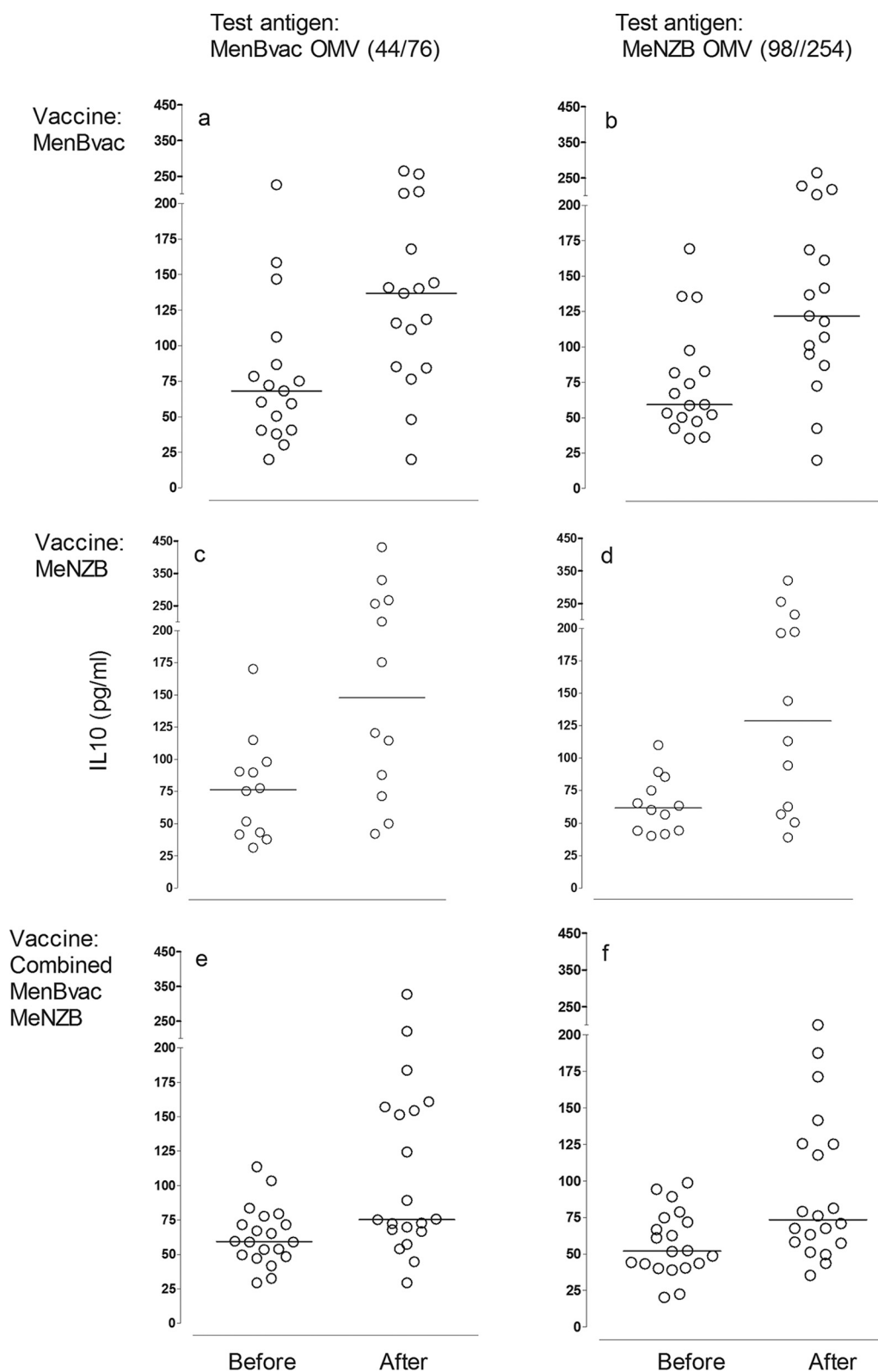


FIG 2 IL-10 responses (pg/ml) as measured by cytometric bead array assay of supernatants from cell cultures (day 6) stimulated *in vitro* with MenBvac and MeNZB OMVs before (week 0) and 6 weeks after the third dose of vaccination with MenBvac alone ($n = 17$), MeNZB alone ($n = 12$), or combined vaccine ($n = 20$). The results are expressed as pg/ml. The P values according to Wilcoxon paired signed-rank test for vaccine-induced IL-10 responses observed in the different vaccine groups were 0.0373 (a), 0.0021 (b), 0.0079 (c), 0.0120 (d), 0.0054 (e), and 0.0011 (f).

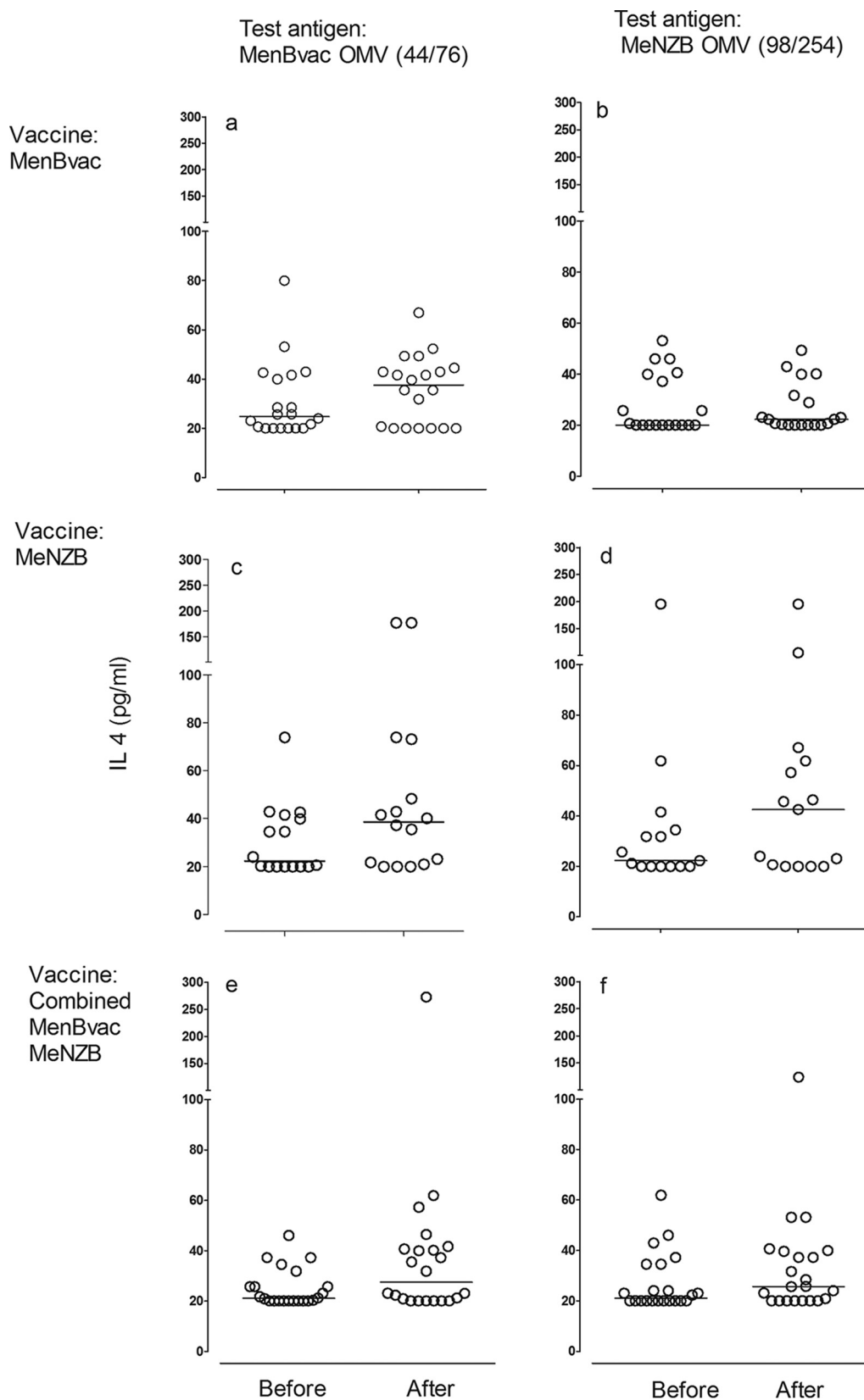


FIG 3 IL-4 responses (pg/ml) as measured by cytometric bead array assay of supernatants from cell cultures (day 6) stimulated *in vitro* with MenBvac and MeNZB OMVs before (week 0) and 6 weeks after the third dose of vaccination with MenBvac alone ($n = 19$ and 20), MeNZB alone ($n = 15$ and 16), or combined vaccine ($n = 22$). The results are expressed as pg/ml. The P values according to Wilcoxon paired signed-rank test for vaccine-induced IL-4 responses observed in the different vaccine groups were 0.2243 (a), 0.2894 (b), 0.0322 (c), 0.0273 (d), 0.0054 (e), and 0.0280 (f).

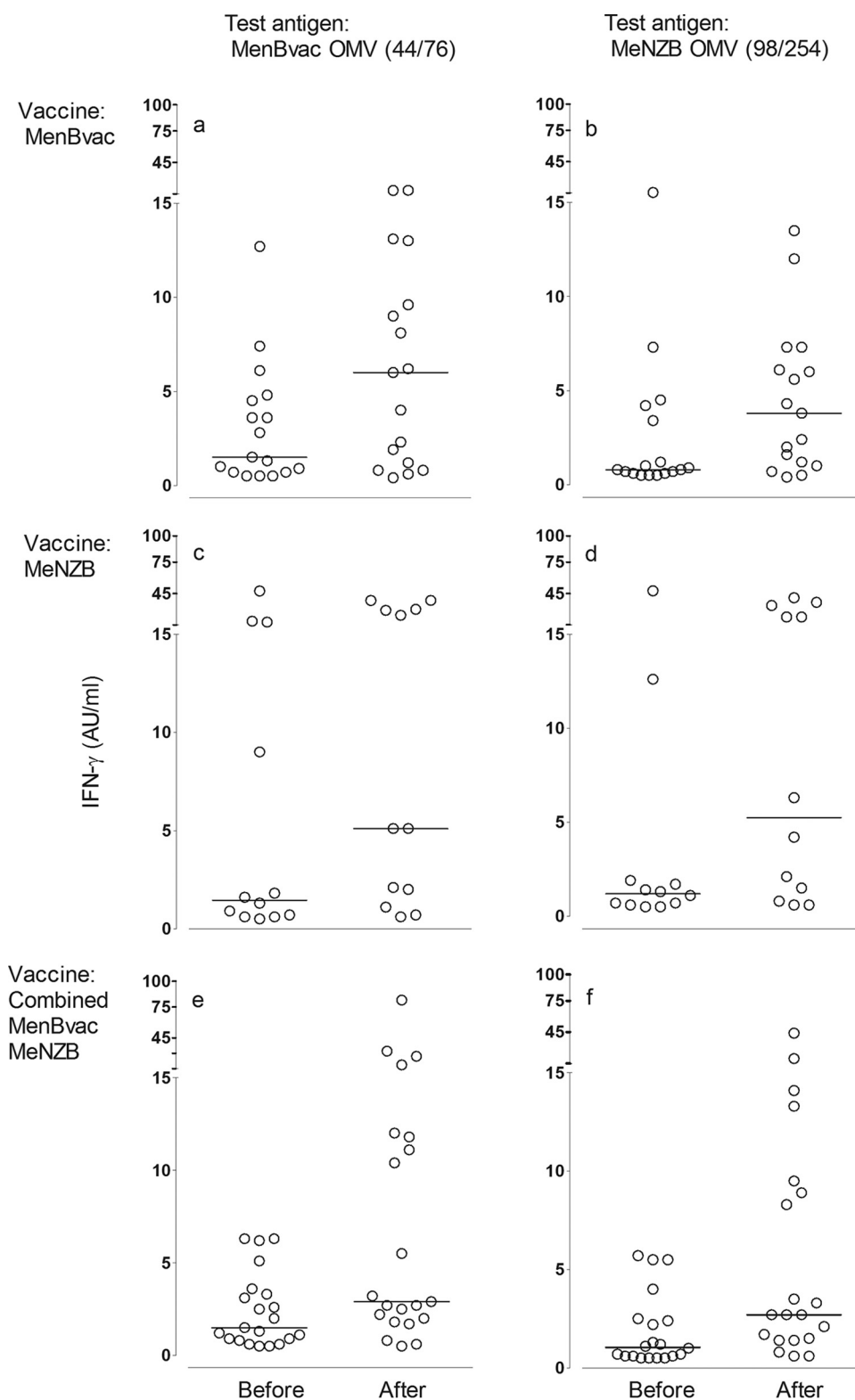


FIG 4 IFN- γ responses as measured by cytometric bead array assay of supernatants from cell cultures (day 6) stimulated *in vitro* with MenBvac and MenNZB OMVs before (week 0) and 6 weeks after the third dose of vaccination with MenBvac alone ($n = 17$), MenNZB alone ($n = 12$), or combined vaccine ($n = 21$ and 20). The results are expressed as arbitrary units (AU)/ml. The P values according to Wilcoxon paired signed-rank test for vaccine-induced IFN- γ responses observed in the different vaccine groups were 0.0179 (a), 0.1183 (b), 0.3203 (c), 0.0376 (d), 0.0012 (e), and 0.0008 (f).

viously used this assay to demonstrate the induction of CD4⁺ T cell responses both after systemic (36) and mucosal (35) vaccination with MenBvac and 44/76 OMVs, respectively. In these studies both OMV (44/76) and PorA specific T cell responses were shown to correlate with vaccine-induced serum IgG, SBA, and nasal IgA responses (35, 36). PorA has been shown to be a major antigen target for both antibodies and T cells in natural and OMV-induced immunity (4, 35–39). Proliferative T cell responses were also observed after vaccination with the Cuban OMV vaccine (40) based on a different serotype (B:4,P1,19,15).

In the present study, we have shown that OMV vaccine-induced T cells also show cross-reactive properties when tested against OMV antigens from heterologous strains with different serotypes. This observation is consistent with information on extensive sequence homology within the major meningococcal antigens recognized by T cells. Testing of overlapping peptides from such antigens, including PorA, has demonstrated the presence of HLA class II-restricted T cell epitopes localized to conserved regions (38, 41–43). In addition, we have demonstrated that cross-reactive T cell responses were accompanied by the production of both Th1 and Th2 cytokines measured as IFN- γ , IL-4, and IL-10. Parenteral immunization with OMV vaccines has previously been claimed to induce a Th1-biased response (40, 44), but a clear association between any defined cytokine profile and protection against meningococcal disease has not yet been established. It has been suggested that a Th1-skewed immune response can be beneficial by promoting enhanced Fc γ R1 expression on phagocytes, neutrophil mobilization, and intracellular bactericidal activity, whereas a more balanced Th1/Th2 response will be necessary to secure the activation and regulation of essential B cell responses such as IgG and IgA production and opsonophagocytosis (45, 46). As an integral part of the Th2 response, IL-10 basically induces naive human B cells expressing surface IgD to switch to IgG1 and IgG3 production, which are the main IgG subclasses contributing to antibody-mediated protection against meningococcal disease (47–49). However, one may also speculate whether the IL-10 response observed here also could indicate the presence of regulatory T cells (50, 51) negatively influencing the duration of antibody levels after OMV vaccination (11, 18).

Although cytokine analysis of supernatants does not give information about the cellular source of the individual cytokines produced, the results at least suggest that a mixed cytokine profile seems to be induced after vaccination. The ideal approach for further addressing both cytokine balance (Th1/Th2) and the multifunctionality of surface marker-defined T cell populations would have been to use multiparameter flow cytometry. Our findings highlight the importance of the complex interplay between humoral and cell-mediated immunity against meningococci and suggest that OMV-based vaccines have the potential to induce both Th1 and Th2 cytokines, at least as detected in cells from peripheral circulation. Natural immunity against meningococci at the systemic level has been shown to be a balanced Th1/Th2 memory response (52), whereas the mucosal T cell response has been characterized as Th1 polarized (39, 44, 52). Hence, the results presented here obtained with OMV vaccination are consistent with the picture seen in natural immunity in adults, at least at the systemic level. Moreover, previous findings that infected young children have less ability to develop a Th2 response than older age groups points to the importance of inducing an appropriate cytokine balance to achieve protection in all age groups (53).

In conclusion, the results demonstrated that proliferative CD4⁺ T cell responses involving both Th1 and Th2 cytokines can be induced to the same extent by different tailor-made OMV vaccines given either separately or in combination with half the dose of each vaccine. The possibility to reduce the amount of antigen used is an important aspect for developing economically viable vaccines. The results are consistent with functional antibody responses detected as SBA and OPA in the same clinical study (18) and strengthen the prospects for using combined OMV vaccines to increase the protective coverage. Improved protection and coverage may also be achieved by developing future recombinant OMV vaccines overexpressing protective antigens from dominant epidemic meningococcal strains (19).

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